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Research paper

Analysis of the immunoproteome of *Mycoplasma mycoides* subsp. *mycoides* small colony type reveals immunogenic homologues to other known virulence traits in related *Mycoplasma* species

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ABSTRACT

Contagious bovine pleuropneumonia (CBPP) caused by *Mycoplasma mycoides* subsp. *mycoides* small colony type (*Mmm*SC) has been eradicated in the developed world, but it is still present in many countries of sub-Saharan Africa. After initially successful control measures in the 1960s it has been spreading due to a lack of money, fragmentation of veterinary services, uncontrolled cattle movement, insufficient vaccine efficacy and sensitivity of current diagnostic tests.

In this study we used two-dimensional polyacrylamide gel electrophoresis followed by immunoblot with sera from *Mmm*SC-infected animals and MALDI-ToF mass spectrometry to identify novel immunogenic proteins as candidate molecules for improved diagnostics and vaccines. We identified 24 immunogens recognized by pooled sera from experimentally infected cattle. Furthermore, a serum from an animal with acute clinical disease as well as severe pathomorphological lesions recognized 13 additional immunogens indicating variation in the antibody responses to CBPP amongst cattle. Most immunogens showed compelling similarity to protein/gene sequences in the two ruminant pathogens *M. capricolum* subsp. *capricolum* and *M. mycoides* subsp. *mycoides* large colony type both belonging to the mycoides cluster. Three of these proteins, namely glycerol-3-phosphate oxidase, adenylosuccinate synthase, and glyceraldehyde-3-phosphate dehydrogenase, had no compelling homologue in the other distantly related bovine pathogen *M. agalactiae*. In addition, translation elongation factor Tu, heat shock protein 70, pyruvate dehydrogenase, and FKBP-type peptidyl-prolyl isomerase, which have been found to mediate adhesion to host tissue in other mycoplasmas were shown to be expressed and recognized by sera. These proteins have potential for the development of improved diagnostic tests and possibly vaccines.

1. Introduction

Contagious bovine pleuropneumonia (CBPP) is regarded as one of the most important livestock diseases in Africa according to the African Union through its Interafrican Bureau for Animal Resources. It affects many cattle stocks especially in sub-Saharan Africa (FAO, 2003)

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and is caused by *Mycoplasma mycoides* subsp. *mycoides* small colony type (*Mmm*SC). Recently, CBPP outbreaks have occurred in Portugal, Spain, France, and Italy and caused massive economic impact in affected regions for several years (Regalla et al., 1996). CBPP is characterised by acute pneumonia, high morbidity, and high mortality when introduced into a naïve herd. Some animals, however, survive acute disease and develop a chronic carrier state, which is characterised by the development of sequestered areas of necrotic tissue in the lung harbouring live *Mmm*SC (Nicholas and Bashiruddin, 1995).

The disease was eliminated from developed countries using a 'stamping-out' policy, movement restriction of affected herds, and vaccination (Newton, 1992). Since a lack of resources for refunding farmers, fragmented veterinary services and ethical beliefs in most parts of Africa strongly object such a stamping-out policy (Thiaucourt et al., 2004), there is an urgent need to improve current control measures. The OIE-recommended diagnostic tests, namely the complement fixation test (CFT) and the competitive-ELISA (cELISA), are hampered by limited sensitivity (Marobela-Raborokgwe et al., 2003). The currently used live vaccines ($T1_{44}$, $T1_{44}$ SR) suffer from a limited duration of protection of only one year and the severe side effects they induce at the inoculation site (Tulasne et al., 1996).

Recently, attempts have been undertaken to elucidate host–pathogen interaction (Bischof et al., 2008; Pilo et al., 2005) and to identify pathogen-specific variable surface proteins (Hamsten et al., 2008), lipoproteins (Abdo et al., 2000; Dedieu et al., 2009; Pilo et al., 2003), and other proteins interacting with the host (March et al., 2006). The *Mycoplasma*-specific codon usage and the very high A + T content of 76% resulting in premature termination and aberrant internal transcription/translation of genomic libraries cloned in *Escherichia coli* have complicated the detection of immunogenic *Mycoplasma* proteins (Minion, 1998). In 2004 the full genome sequence of the *M. mycoides* subsp. *mycoides* SC type strain PG1 was published (Westberg et al., 2004) and paved the way for the combination of two-dimensional gel electrophoresis (2-DE), immunoblot, and mass spectrometry (MS) as a means to detect immunogenic proteins.

This manuscript describes the application of 2-DE, immunoblot and MS to detect immunogenic proteins, which might be useful for the development of next generation vaccines and diagnostics. Additionally, a sequence comparison of identified immunogens with the two closely related ruminant *Mycoplasma* species *M. mycoides* subsp. *mycoides* large colony type and *M. capricolum* subsp. *capricolum* (Manso-Silvan et al., 2007) as well as the bovine pathogen *M. agalactiae* PG2 was performed to assess their specificity.

2. Materials and methods

2.1. Bacterial strains and culture conditions

The *Mmm*SC type strain $PG1^T$ was grown as stationary culture for 3 days at 37 °C in 1000 ml bottles in modified PH media (Kirchhoff and Rosengarten, 1984), supplemented with 20% horse serum (WDT, Garbsen, Germany). The cells were then centrifuged at 20,000 × *g* for 30 min at 4 °C and washed three times in phosphate-buffered saline (PBS; 150 mM NaCl, 1.5 mM KH_2PO_4, 9 mM $Na_2HPO_4 \times 12H_2O$, 2.5 mM KCl [pH 7.2]). Washed cell pellets were stored at −20 °C.

2.2. Raising defined sera

A group of 15 cattle was infected as described previously (Jores et al., 2008). Briefly, zebu cattle (*Bos indicus*) three years of age were inoculated intrabroncheally with 60 ml of culture of *Mmm*SC B237 (5×10^7 colony forming units/animal) followed by 20 ml of 1.5% warm agar and 30 ml of PBS. The animals were monitored daily for clinical signs and sacrificed 29–47 days post-infection. Serum samples were taken, and post mortem examination was performed on the day of slaughter. The sera were irradiated with 25 kilo gray using a caesium (137Cs) source before being shipped to Germany for further analysis.

2.3. Two-dimensional gel electrophoresis and immunoblot

Protein sample preparation and two-dimensional gel electrophoresis (2-DE) were essentially performed as described previously (Buettner et al., 2008). Briefly, frozen *Mmm*SC $PG1^T$ cell pellets obtained from 1000 ml of culture volume were thawed and resuspended in 1 ml of PBS and ruptured using 250 mg of zirconium beads (0.1 mm diameter, Roth, Karlsruhe, Germany) in a FastPrep® Instrument (Qbiogene, Heidelberg, Germany) three times for 40 s at intensity setting 5.0. Cell debris was removed by repeated centrifugation (15,000 × *g* at 4 °C for 30 min), the protein concentration of the remaining supernatant was determined using the MicroBC® 146 assay (Uptima Interchim, Montlucon Cedex, France), and confirmed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE). For 2-DE, proteins were precipitated with trichloroacetic acid (TCA; 10% final concentration), washed with acetone, and solubilized in lysis buffer (30 mM Tris–HCl [pH 8.0], 7 M urea [Roth, Karlsruhe, Germany], 2 M thiourea [Sigma], and 4% [wt/vol] CHAPS [Roth]) to a protein concentration of 5 mg/ml). First dimension focusing was performed on 24 cm non-linear pH gradient (3–11 NL) strips (GE Healthcare, Darmstadt, Germany) for 21 h using the following program: 3 h at 150 V, 3 h at 300 V, 6 h at a 1000 V gradient, 3 h at an 8000 V gradient, and 6 h at 8000 V. The second dimension was carried out on 12.5% SDS PAGE gels at 12 °C and 50 V for 3 h followed by 100 V for 15 h using the Ettan™ DaltSix Electrophoresis System (GE Healthcare). All experiments were run in triplicate. Proteins on the first gel were visualized by staining with colloidal Coomassie (Candiano et al., 2004) or by a modified protocol of the silver staining method of Blum (Rabilloud, 1999). The proteins of the remaining two gels were electro-transferred by using Multiphor II (GE Healthcare) to nitrocellulose membranes BA85 (Schleicher und Schüll, Dassel, Germany) for immunoblot analyses. Immunoreactive proteins were detected as described elsewhere (Meens et al., 2006). Briefly, serum from animal 543, as well as a pool of sera from cattle with pathomorphological lesions (505, 509, 513, 519, 522, 525,